PATENT
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MANIPULATION OF THE PHENOLIC ACID CONTENT AND DIGESTIBILITY OF PLANT CELL WALLS BY TARGETED EXPRESSION OF GENES ENCODING CELL WALL DEGRADING ENZYMES

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CROSS-REFERENCE TO RELATED APPLICATIONS

Pursuant to 35 U.S.C. §119(e), the present application claims benefit of and priority to USSN 60/249,608, entitled "MANIPULATION OF THE PHENOLIC ACID CONTENT AND DIGESTIBILITY OF FORAGE GRASS CELL WALLS BY TARGETED EXPRESSION OF A FERULIC ACID ESTERASE GENE", filed November 17, 2000, by Morris et al.

FIELD OF THE INVENTION

This invention relates to methods to enhance to availability of fermentable carbohydrates.

BACKGROUND OF THE INVENTION

The present crisis in livestock agriculture has prompted a resurgence of interest in grass-fed animals. However, while a high-forage diet may be desirable, it does not currently satisfy the demands of modern animal production. For the animal to make efficient use of the forage it consumes, the energy demands of the micro-organisms in the rumen must be met and synchronized with the availability of plant proteins. Otherwise this lack of synchrony will lead to (a) proteins and other nutrients being poorly utilized in the rumen, (b) loss of nitrogen, in urine and feces

and therefore, the environment and (c) the need to feed excessive amounts of protein concentrates as supplements to the ruminant diet.

Cellulose and hemicellulose in grass and maize tissues could meet the energy requirements of the ruminant or provide new feed-stocks for industrial fermentation to ethanol. This potential is not currently realized because the cell walls are lignified and the cell wall polysaccharides highly cross-linked with phenolic residues and lignin, resulting in low rates of plant cell wall digestion in comparison to rates of protein breakdown in ruminants. This is a particular problem for the most important forages in Europe, the ryegrasses *Lolium perenne* and *L. mutiflorum* as well as one of the major impediments to the wider use of better adapted species, such as *Festuca arundinacea*, as a forage crop. Increasing the digestibility index of grasses has therefore been a major breeding objective for several decades but progress has been slow due to difficulties in fixing natural variation in the synthetic varieties derived from these outbreeding species (Hayward, *et al., TAG* **70**:48 (1985)).

Removing labile phenolics by chemical treatment with alkali is known to increase the biodegradability and nutritional value of low-quality feed such as cereal straw, and is employed commercially for feed upgrading. Reducing phenolic cross-linking of cell wall carbohydrates is therefore a predictable way of improving the rate of digestion and digestibility of ryegrass. However chemical modification may have other disadvantages. Therefore, genetic modification would be a preferable method of changing the cell wall chemistry of highly digestible varieties. Many in the field are pursuing this approach. An alternative, however, is to use genetic modification to reduce the levels of phenolic acids in the cell walls available for crosslinking either by directly disrupting ester bonds linking phenolics and lignins to cell wall polysaccharides or by preventing excessive ferulation of cell wall carbohydrates prior to their incorporation into the cell wall.

This invention meets this and other needs by using targeted or inducible expression of cell wall degrading enzymes in plants.

SUMMARY OF THE INVENTION

Provided herein are methods for enhancing the availability of fermentable carbohydrates. In one aspect, there is provided an expression cassette comprising a DNA sequence encoding at least one cell wall degrading enzyme. The DNA sequence encoding at least one cell wall degrading enzyme may be operatively linked to a promoter sequence. The promoter may be constitutive or inducible. The expression cassette may further comprise a targeting sequence.

In one embodiment, the cell wall degrading enzyme is selected from the group consisting of ferulic acid esterase, xylanase, xylosidase, cellulase, endoglucanase, and cellbiohydrolase. In a preferred embodiment cell wall degrading enzyme is derived from a fungal source. In a more preferred embodiment, the fungal ferulic acid esterase is an *Aspergillus* ferulic acid esterase, preferably *A. niger*. In another embodiment the xylanase is derived from *Trichoderma*, preferably *T. reesei*.

In another aspect of the invention, there is provided a plant transformed with the expression cassette comprising a DNA sequence encoding at least one cell degrading enzyme. The plant may be selected from the group consisting of Festuca, Lolium, Avena and Zea. In a preferred embodiment the plant is a forage grass. In another embodiment, the plant is maize.

Further provided herein is a method of controlling the level of phenolic acids in plant cell walls of a transgenic plant. The method, in one embodiment, comprises introducing to a plant cell an expression cassette comprising a DNA sequence encoding at least one cell wall degrading enzyme, preferably a ferulic acid esterase.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the scope and spirit of the invention will become apparent to one skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 illustrates a restriction map of a DNA fragment containing the gene encoding the 38kd ferulic acid esterase.

Figures 2 A-E illustrate the complete DNA (SEQ. ID NO:__), with highlighting to point out the signal sequence, intron and various restriction endonuclease sites, and amino acid sequence (SEQ. ID. NO:__) corresponding to the gene encoding the 38 kD ferulic acid esterase isolated from *Aspergillus niger.*

Figure 3 illustrates the DNA sequence of the gene encoding the 38 kD esterase (SEQ. ID. NO:__).

Figure 4 illustrates the construction of the intronless ferulic acid esterase isolated from *Aspergillus niger.*

Figure 5 illustrates that the overlapping of PCR products made with primers FAE-I5 and FAE-I3 creates two possible uninterrupted reading frames – the top in the figure below is functional (highlighted serine is at active site), the bottom is inactivated.

Figure 6 illustrates the possible vector constructions useful in the present invention. Various combinations are possible. Although and FAE gene is depicted another cell wall degrading enzyme may be used alone (i.e., instead of) or in conjunction with the FAE gene. Amp = ampicillin resistance gene.

Figure 7 illustrates pCOR105.

Figure 8 illustrates a generic ALE-TER vector.

Figure 9 illustrates the KDEL-COOH ER retention sequences.

Figure 10 illustrates the FAE-LINKER-FRAMESHIFT structure and sequence.

Figure 11 illustrates plant transformation cassettes.

Figure 12 is a table of the vectors used herein.

Figure 13 depicts the barley aleurain vacuolar and apoplast signal sequence.

Figure 14 illustrates the rat sialyl transferase structure and sequence.

Figure 15 illustrates the potato protease inhibitor II (PPI) motif structure and sequence.

Figure 16 illustrates the targeted expression of gfp to different cell compartment. Also shown are schematics of the vectors used.

Figure 17 illustrates the FAE activity in transgenic *Festuca arundinacea* leaves of different ages under ER and APO targeting sequences.

Figure 18 illustrates the FAE activity in transgenic *Festuca arundinacea* leaves of different ages under Vac targeting sequence.

Figure 19 illustrates the FAE activity in transgenic *Lolium mutflorum* leaves of different ages.

Figure 20 illustrates the FAE activity in transgenic *Lolium mutflorum* leaves under Vac, ER and APO targeting sequences.

Figure 21 illustrates the levels of esterified monomeric and dimeric hydroxycinnamic acids in *Festuca arundinacea* plants expressing FAE under Vac targeting sequence.

Figure 22 illustrates the levels of esterified monomeric and dimeric hydroxycinnamic acids in *Festuca arundinacea* plants expressing FAE under APO and ER targeting sequence.

Figure 23 illustrates the *in vitro* dry matter digestibility of leaf tissue of mature *Festuca arundinacea* plants expressing FAE under an actin promoter.

Figure 24 illustrates the *in vitro* dry matter digestibility of leaf tissue of mature *Lolium mutflorum* plants expressing FAE under an actin promoter.

Figure 25 illustrates the rate of fermentation and cumulative gas production in *Festuca arundinacea* cells.

Figure 26 illustrates the in vitro fermentation of *Festuca arundinacea* cell walls from cell cultures expressing recombinant FAE1.

Figure 27 illustrates the Time to maximum rate digestion for *Festuca arundinacea* cells.

Figure 28 illustrates the total gas production in *Festuca arundinacea* cells.

Figure 29 illustrates the kinetics of FAE activity by ferulic acid release from cell wall under self digestion in *Festuca arundinacea* and stimulation by xylanase.

Figure 30 illustrates the beta-glucoronidase activity under the Lolium See1 senescence promoter in leaves of transgenic plants of *Lolium mutflorum.*

Figure 31 illustrates the release of monomeric and dimeric HCAs on self digestion of leaves of vacuolar targeted FAE expressing plants.

Figure 32 is a schematic of the pTP10-1 vector. Also shown is the 5338 bp nucleotide sequence of the vector.

Figure 33 is a schematic of the pUA4-4 vector. Also shown is the 5345 bp nucleotide sequence of the vector.

Figure 34 is a schematic of the pTU4 vector. Also shown is the 5337 bp nucleotide sequence of the vector.

Figure 35 is a schematic of the pTT5.14 vector. Also shown is the 5395 bp nucleotide sequence of the vector.

Figure 36 is a schematic of the pTP8-5 vector. Also shown is the 5337 bp nucleotide sequence of the vector.

Figure 37 is a schematic of the pTP5-1 vector. Also shown is the 5277 bp nucleotide sequence of the vector.

Figure 38 is a schematic of the pTP4a2 vector. Also shown is the 5327 bp nucleotide sequence of the vector.

Figure 39 is a schematic of the pTP3-1 vector. Also shown is the 5338 bp nucleotide sequence of the vector.

Figure 40 is a schematic of the pTU5 vector. Also shown is the 5337 bp nucleotide sequence of the vector.

Figure 41 is a schematic of the pGT6 vector. Also shown is the 4773 bp nucleotide sequence of the vector.

Figure 42 is a schematic of the pJQ5 vector. Also shown is the 5034 bp nucleotide sequence of the vector.

Figure 43 is a schematic of the pJO6.1 vector. Also shown is the 4950 bp nucleotide sequence of the vector.

Figure 44 is a schematic of the pJQ4 vector. Also shown is the 4974 bp nucleotide sequence of the vector.

Figure 45 is a schematic of the pPQ10.1 vector. Also shown is the 5164 bp nucleotide sequence of the vector.

Figure 46 is a schematic of the pJQ3 vector. Also shown is the 4965 bp nucleotide sequence of the vector.

Figure 47 is a schematic of the pUG4 vector. Also shown is the 5295 bp nucleotide sequence of the vector.

Figure 48 is a schematic of the pUB8.11 vector. Also shown is the 5001 bp nucleotide sequence of the vector.

Figure 49 is a schematic of the pTP11-1 vector. Also shown is the 5387 bp nucleotide sequence of the vector.

Figure 50 illustrates the actin promoter and its corresponding nucleotide sequence.

Figure 51 illustrates the Aleurain-NPIR delete structure. The corresponding nucleotide sequences are also shown.

Figure 52 illustrates the SEE1 (senescence enhanced) promoter sequence.

Figure 53 illustrates the SEE1 (senescence enhanced) promoter sequence plus the vacuolar aleurain signal/NPIR sequence.

DETAILED DESCRIPTION OF THE INVENTION

The invention will now be described in detail by way of reference only using the following definitions and examples. All patents and publications, including all sequences disclosed within such patents and publications, referred to herein are expressly incorporated by reference.

Unless defined otherwise herein, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Singleton, *et al.*, DICTIONARY OF MICROBIOLOGY AND MOLECULAR BIOLOGY, 2D ED., John Wiley and Sons, New York (1994), and Hale & Marham, THE HARPER COLLINS DICTIONARY OF BIOLOGY, Harper Perennial, NY (1991) provide one of skill with a general dictionary of many of the terms used in this invention. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described. Numeric ranges are inclusive of the numbers defining the range. Unless otherwise indicated, nucleic acids are written left to right in 5' to 3' orientation; amino acid sequences are written left to right in amino to carboxy

orientation, respectively. Practitioners are particularly directed to Sambrook *et al.,* 1989, and Ausubel FM *et al.,* 1993, for definitions and terms of the art. It is to be understood that this invention is not limited to the particular methodology, protocols, and reagents described, as these may vary.

The headings provided herein are not limitations of the various aspects or embodiments of the invention which can be had by reference to the specification as a whole. Accordingly, the terms defined immediately below are more fully defined by reference to the specification as a whole.

Definitions

It should be noted that, as used in this specification and the appended claims, the singular forms "a," "an, "and "the" include plural referents unless the content clearly dictates otherwise. Thus, for example, reference to a composition containing "a compound" includes a mixture of two or more compounds. It should also be noted that the term "or" is generally employed in its sense including "and/or" unless the content clearly dictates otherwise.

"Conservatively modified variants" applies to both amino acid sequences and polynucleotides. With respect to particular polynucleotides, conservatively modified variants refers to those polynucleotides that encode identical or essentially identical amino acid sequences, or where the polynucleotide does not encode an amino acid sequence, to essentially identical sequences. Because of the degeneracy of the genetic code, a large number of functionally identical polynucleotides encode any given protein. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations," which are one species of conservatively modified variations. Every polynucleotide herein which encodes a polypeptide also describes every possible silent variation of the nucleic acid. One of skill will recognize that each codon in a polynucleotide (except AUG, which is ordinarily the only codon for methionine) can be modified to yield a functionally identical molecule. Accordingly, each silent variation of a polynucleotide which encodes a polypeptide is implicit in each described

sequence. For purposes of protein expression, there are "sub-optimal codons." These are codons that are not preferred by a particular genus or species. Altering these "sub-optimal codons" to "preferred codons" is a silent mutation in that the amino acid encoded by the codons is the same but one codon is preferentially expressed by the particular genus, *e.g., Triticum spp.*

As to amino acid sequences, one of skill will recognize that individual substitutions, deletions or additions to a polynucleotide, peptide, polypeptide, or protein sequence which alters, adds or deletes a single amino acid or a small percentage of amino acids in the encoded sequence is a "conservatively modified variant" where the alteration results in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art.

The following six groups each contain amino acids that are conservative substitutions for one another:

1) Alanine (A), Serine (S), Threonine (T);
2) Aspartic acid (D), Glutamic acid (E);
3) Asparagine (N), Glutamine (Q);
4) Arginine (R), Lysine (K);
5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and
6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

(*see, e.g.*, Creighton, *Proteins* (1984)).

"Pyroglutamic acid" is the cyclized internal amide of L-glutamic acid

The phrase "controlling the level of phenolic acids" refers to the manipulation of phenolic acid expression in plants, particularly plant cell walls. The manipulation can be either positive; *e.g.,* increasing the levels of phenolic acids; negative, *e.g.,* decreasing the level of phenolic acids; or neutral, *e.g.,* changing the relative amounts of specific phenolic acids in the cell walls but keeping the total amount relatively the same. The timing of manipulation can be during plant growth or after plant growth, *e.g.,* after a plant has been cut or pulled from the ground or ingested. "Plant cell walls" refers to the cell walls of any cell of the plant.

The term "derived" means that a polynucleotide or protein is related to

another polynucleotide or protein. The relations can be one of homology, *e.g.,* nucleotides and proteins from certain species are homologous to similar polynucleotides and proteins of other species; analogy, *e.g.,* proteins perform the same function and therefore are related to each other regardless of organism of origin. The relationship can be a man-made one, *e.g.,* a protein (and a polynucleotide) can be derived from another protein by mutation; or chemical manipulation (peptidomimetics). Furthermore, a protein or a polynucleotide can be derived from an organism if, in the natural state, the protein or polynucleotide is found in one organism but recombinantly produced in another.

The term "exogenous polynucleotide" refers to a polynucleotide which is introduced into the plant by any means other than a sexual cross or sexual reproduction. Examples of means by which this can be accomplished are described below, and include *Agrobacterium*-mediated transformation, biolistic methods, electroporation, *in planta* techniques, and the like. Such a plant containing the exogenous polynucleotide is referred to here as an R_1 generation transgenic plant. Transgenic plants which arise from sexual cross or by selfing are progeny of such a plant.

The term "isolated polynucleotide molecule" or "isolated protein" refers to a polynucleotide or protein which is essentially free of other cellular components with which it is associated in the natural state. It is preferably in a homogeneous state although it can be in either a dry or aqueous solution. Purity and homogeneity are typically determined using analytical chemistry techniques such as polyacrylamide gel electrophoresis or high performance liquid chromatography. A protein which is the predominant species present in a preparation is substantially purified. In particular, an isolated *FAE1* gene is separated from open reading frames which flank the gene and encode a protein other than FAE1.

A *"FAE1* encoding polynucleotide" is a nucleic acid sequence comprising (or consisting of) a coding region of an FAE 1 gene or which encodes a FAE1 polypeptide. *FAE1* polynucleotides can also be identified by their ability to hybridize under low stringency conditions (see below) to nucleic acid probes having a sequence of 8 to 300 bases, preferably a sequence of 80 to 100 bases

in the sequence disclosed in WO 98/14594.

The term "nucleic acid encoding," "nucleic acid sequence encoding" or "polynucleotide encoding" refers to a polynucleotide which directs the expression of a specific protein or peptide. The polynucleotides include both the DNA strand sequence that is transcribed into RNA and the RNA sequence that is translated into protein. The polynucleotides include both full length polynucleotides as well as shorter sequences derived from the full length sequences. It is understood that a particular polynucleotide includes the degenerate codons of the native sequence or sequences which may be introduced to provide codon preference in a specific host cell. The polynucleotide includes both the sense and antisense strands as either individual single strands or in the duplex form.

The term "operably linked" refers to functional linkage between a promoter and a second sequence, wherein the promoter sequence initiates transcription of RNA corresponding to the second sequence.

The term "plasmid" refers to a circular double stranded DNA molecule which comprises the coding sequence of interest, regulatory elements, a selection marker and optionally an amplification marker. A plasmid can transform prokaryotic cells or transfect eukaryotic cells. An "expression cassette" means a portion of a plasmid (or the entire plasmid) containing the regulatory elements desired for transcription, translation and/or expression and the coding region of a polynucleotide. A plasmid may contain one or more expression cassettes. If multiple expression cassettes are introduced into a plant, they can be introduced simultaneously or at different times. If simultaneous introduction is desired, the expression cassettes can be on one plasmid or more. Typically, an expression cassette comprises a promoter, poly A+ tail, and signal sequences that target the expressed polypeptide to a specific region of a cell or to be secreted, if desired. Examples of signal sequences that "target expression" of ferulic acid esterase include sequences located upstream of the FAE coding sequence. The polynucleotide that encodes the signal sequence is found preferably within the 100 nucleotides "upstream" (in the 5' direction) from the initiation codon (AUG). More preferably, the polynucleotide that encodes the signal sequence is found within the 50 nucleotides upstream from the initiation

codon. Many different cellular organelles are targeted by the signal sequences used in this invention. The organelles include, but are not limited to, vacuoles, Golgi apparati, endoplasmic reticula, and apoplasts. In addition to upstream signal sequences, the expression cassette of this invention may include a polynucleotide that encodes a signal sequence at the 3' end. These signal sequences include, but are not limted to stop codons and the KDEL sequence. In addition to KDEL, other similar sequences are contemplated by this invention, including but not limited to RDEL. In addition to a KDEL sequence, a signal sequence can include a linker to a KDEL sequence. A linker is an extension of the reading frame of the encoding polynucleotide to the signal sequence. Preferably, the polynucleotide encoding the signal sequence is directly downstream from the coding sequence, more preferably less than 100 base pairs from the stop codon, more preferably less than 20 base pairs from the stop codon.

The term "polynucleotide," "polynucleotide" or "nucleic acid sequence" refers to deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form. Unless specifically limited, the term encompasses polynucleotides containing known analogues of natural nucleotides which have similar binding properties as the reference polynucleotide and are metabolized in a manner similar to naturally occurring nucleotides. Unless otherwise indicated, a particular *FAE1* polynucleotide of this invention also implicitly encompasses conservatively modified variants thereof (*e.g.* degenerate codon substitutions) and complementary sequences and as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer *et al.*, *Nucleic Acid Res.* **19**:5081 (1991); Ohtsuka *et al.*, *J. Biol. Chem.* **260**:2605-2608 (1985); and Cassol *et al.*, 1992; Rossolini *et al.*, *Mol. Cell. Probes* **8**:91-98 (1994)). The term polynucleotide is used interchangeably with gene, cDNA, and mRNA encoded by a gene.

The term "polypeptide," "peptide," and "protein" are used interchangeably and refer to amino acids connected by peptide bonds. Polypeptides can be

entire proteins or portions thereof. For Example. a FAE1 polypeptide may refer to the entire FAE1 protein or fragments of the FAE1 protein. A "ferulic acid esterase with an altered glycosylation site" refers to a FAE protein wherein a mutation has changed the glycosylation pattern of the protein. Mutations that effect such changes are well known in the art and include, but are not limited to, amino acid substitutions, and mutations in the proteins of the Golgi apparatus and endoplasmic reticulum that effect glycosylation of proteins.

The term "promoter" refers to a polynucleotide that directs expression of a coding sequence. A promoter can be constitutive, *i.e.,* relatively independent of the stage of differentiation of the cell in which it is contained or it can be inducible, *i.e.,* induced be specific environmental factors, such as the length of the day, the temperature, *etc.* or a promoter can be tissue-specific, *i.e.,* directing the expression of the coding sequence in cells of a certain tissue type. A "senescence" promoter is an inducible promoter that causes transcription to be initiated upon a certain event relating to age of the organism. A "heat shock promoter" is an inducible promoter that causes transcription to be initiated upon a change in temperature. An example of a heat shock protein promoter is the Soybean Gmhsp promoter. In addition to these inducible promoters, one of skill will realize that other inducible promoters can be used. For example, a wound induced promoter, like LAP. See, US Patent No. 5,962,670.

The term "purified" denotes that a polynucleotide or protein gives rise to essentially one band in an electrophoretic gel. Particularly, it means that the polynucleotide or protein is at least 85% pure, more preferably at least 95% pure, and most preferably at least 99% pure.

The term "specifically hybridizes" refers to a nucleic acid probe that hybridizes, duplexes or binds to a particular target DNA or RNA sequence when the target sequences are present in a preparation of total cellular DNA or RNA. "Complementary" or "target" nucleic acid sequences refer to those nucleic acid sequences which selectively hybridize to a nucleic acid probe. Proper annealing conditions depend, for example, upon a probe's length, base composition, and the number of mismatches and their position on the probe, and must often be determined empirically. For discussions of nucleic acid probe design and

annealing conditions, see, for example, Sambrook *et al.*, MOLECULAR CLONING: A LABORATORY MANUAL (2ND ED.), Vols. 1-3, Cold Spring Harbor Laboratory, (1989) ("Sambrook") or CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, F. Ausubel *et al.*, ed. Greene Publishing and Wiley-Interscience, New York (1987) ("Ausubel").

The term "stringent conditions" in the context of polynucleotide hybridization experiments such as Southern and northern hybridizations refers to sequence dependent, binding and washing environments. An extensive guide to the hybridization of polynucleotides is found in Tijssen (1993) LABORATORY TECHNIQUES IN BIOCHEMISTRY AND MOLECULAR BIOLOGY--HYBRIDIZATION WITH NUCLEIC ACID PROBES part I chapter 2 "overview of principles of hybridization and the strategy of nucleic acid probe assays", Elsevier, New York. Generally, highly stringent hybridization and wash conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Very stringent conditions are selected to be equal to the T_m for a particular probe. An example of stringent hybridization conditions for hybridization of complementary polynucleotides which have more than 100 complementary residues on a filter in a Southern or northern blot is 50% formalin with 1 mg of heparin at between 40 and 50°C, preferably 42°C, with the hybridization being carried out overnight. An example of highly stringent wash conditions is 0.15M NaCl at from 70 to 80°C with 72°C being preferable for about 15 minutes. An example of stringent wash conditions is a 0.2x SSC wash at about 60 to 70°C, preferably 65°C for 15 minutes (*see,* Sambrook, *supra* for a description of SSC buffer). Often, a high stringency wash is preceded by a low stringency wash to remove background probe signal. An example medium stringency wash for a duplex of, *e.g.,* more than 100 nucleotides, is 1x SSC at 40 to 50°C, preferably 45°C for 15 minutes. An example low stringency wash for a duplex of, *e.g.,* more than 100 nucleotides, is 4-6x SSC at 35 to 45°C, with 40°C being preferable, for 15 minutes. In general, a signal to noise ratio of 2x (or higher) than that observed for an unrelated probe in the particular hybridization assay indicates detection of a specific hybridization. Polynucleotides which do

not hybridize to each other under stringent conditions are still substantially identical if the polypeptides which they encode are substantially identical. This occurs, *e.g.*, when a copy of a polynucleotide is created using the maximum codon degeneracy permitted by the genetic code.

The term "transgenic plant" refers to a plant into which exogenous polynucleotides have been introduced and their progeny. Typically, cells of a plant are transformed with the exogenous polynucleotide and a transgenic plant is regenerated from the transformed cells. The regenerated plant is then bred to produce a strain of transgenic plants.

"Xylanase" (EC 3.2.1.8) refers to a well described class of gylcosyl hydrolases that hydrolize xylan. Commercial applications of xylanase include the degradation and bleaching of wood pulp for paper making. Xylanase can also be added to animal feed to improve the digestibility of plant matter. Typically, commercial xylanase is derived from fungi. A preferred xylanase is derived from *Trichoderma.*

Preferred Embodiments

Plant cell walls contain a range of alkali-labile ester-linked phenolic acids. In particular, grass cell walls are characterized by the presence of large amounts of esterified ferulic and p-coumaric acids (mainly in their E configurations), linked to arabinoxylans at the C5 of arabinose. These are released as ferulated oligosaccharides (FAX and PAX) by cellulase treatment but *in vivo* provide a substrate for peroxidase-catalyzed cross-linking of cell wall polysaccharides and lignin. The high levels of these phenolic acids and their dimers have a dramatic influence on the mechanical properties, digestibility and rates of digestion of grasses by ruminants.

Previous work has shown that ferulic acid is the predominant p-hydroxycinnamic acid esterified to grass polysaccharide but until recently the only ferulic acid dehydrodimer to have been isolated was 5,5'-diferulic acid. Recently new dehydrodiferulate dimers and cyclobutane-type dimer mixtures have been isolated from plant cell walls (Waldron, *et al., Phytochemical Analysis* **7**:305 (1996)). As can be seen in Figure 1, these mixtures are present in large amounts in grass cells. Ether linked ferulic acid-coniferyl alcohol dimers, have also been isolated from cell walls (Jacquet, *et al., Polyphenol Comm. Bordeaux*

pp451 (1996)) establishing for the first time that ferulate esters are oxidatively co-polymerized with lignin precursors which may anchor lignins to cell wall polysaccharides. The yield of these dimers in grass cells indicates that phenolic dehydrodimer cross-linking of cell wall polysaccharides is much more extensive than was previously thought.

An enzyme system has been reported from parsley endomembranes that catalyses the ferulation of endogenous polysaccharide acceptors from feruloyl CoA, pointing to the ER/golgi as the site of polysaccharide esterification and the CoA ester as the physiological co-substrate (Meyer, *et al., FEBS Lett.* **290**:209 (1991)). Further evidence for this has been found in water-soluble extracellular polysaccharides excreted in large amounts into the medium by grass cell cultures. This material is highly esterified with ferulic and p-coumaric acid at levels similar to the cell walls of the cultured cells.

Feruloyl esterase activity has been detected in several fungal species including, anaerobic gut fungi, yeasts, actinomycetes, and a few fiber-degrading ruminal bacteria, which enables them to de-esterify arabinoxylans and pectins.

Two ferulic acid esterases (FAE), distinguished on the basis of molecular weight and substrate specificity, have been isolated from *Aspergillus niger* and have been shown to quantitatively hydrolyze ferulic acid and release dehydrodiferulate dimers from plant cell walls. Furthermore, FAE has been observed to act synergistically with xylanase to release ferulic acid from plant cell walls at a higher rate. Recently, a ferulic acid esterase (FAE) gene has been cloned from *Aspergillus niger* (Michelson, *et.al.* European Patent Application No. 9510370.1). The inventors have found the recombinant enzyme releases ferulic acid and diferulate dimers from grass cell walls in a concentration dependent manner and that this enzyme is stable at 30°C pH 5.0 in the presence of substrate and has a half life of 61 h at 30°C in the presence of vacuolar extracts (pH 4.6) of grass cells. This gene was, therefore, a candidate for targeted and indicible expression of FAE in grasses (*e.g., Lolium multiflorum*).

The present invention provides for methods of changing the cell wall structure of transgenic plants and therefore, making them more digestible. The method comprises introducing a ferulic acid esterase coding sequence into the

cells of a plant. Operably linked to the coding seqeunce is a promoter that can be either constitutive or inducible and signal sequences that serve to target expression of the coding sequence in the desired organelle in the desired cell of the plant. The signal sequences can be either or both N terminal or C terminal sequences.

Optionally, a second and/or third coding sequence is introduced into the plant. It is preferred that a fungal xylanase coding sequence be coexpressed with the FAE coding sequence. .

This invention also provides for transgenic plants which contain FAE1 coding sequences, leading to more digestible grasses.

Generally, the nomenclature and the laboratory procedures in recombinant DNA technology described below are those well known and commonly employed in the art. Standard techniques are used for cloning, DNA and RNA isolation, amplification and purification. Generally enzymatic reactions involving DNA ligase, DNA polymerase, restriction endonucleases and the like are performed according to the manufacturer's specifications. Basic texts disclosing the general methods of use in this invention include Sambrook, *et al.*, MOLECULAR CLONING, A LABORATORY MANUAL , 2ND ED. (1989); Kriegler, GENE TRANSFER AND EXPRESSION: A LABORATORY MANUAL (1990); and Ausubel *et al.*, (eds.), CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (1994)).

A. Isolation of Polynucleotides

The isolation of the polynucleotides, *e.g.,* FAE1 and xylanase of the invention may be accomplished by a number of techniques. See, for example, copending US application 08/952,445 which describes the isolation of a FAE from *Aspergillus niger*; and copending US application 09/658,772 which describes the isolation of a xylanase from T. reesei.

For instance, oligonucleotide probes based on the sequences cited here can be used to identify the desired gene in a cDNA or genomic DNA library from a desired species. To construct genomic libraries, large segments of genomic DNA are generated by random fragmentation, *e.g.,* using restriction endonucleases, and are ligated with vector DNA to form concatemers that can be

packaged into the appropriate vector. To prepare a library of cDNA from a specific cell culture, *e.g., Aspergillus niger*, mRNA is isolated from the culture and a cDNA library containing the gene transcripts is prepared from the mRNA.

The cDNA or genomic library can then be screened using a probe based upon the sequence of a known polynucleotide such as the polynucleotides cited here. Probes may be used to hybridize with genomic DNA or cDNA sequences to isolate homologous genes in the same or different plant species. In addition to probes derived from known polynucleotides, degenerate probes may be used. Techniques for making and using degenerate probes are well known in the art and can be found in Sambrook and Ausubel.

Alternatively, the polynucleotides of interest can be amplified from polynucleotide samples using amplification techniques. For instance, polymerase chain reaction (PCR) technology can be used to amplify the sequences of the genes directly from mRNA, from cDNA, from genomic libraries or cDNA libraries. PCR and other *in vitro* amplification methods may also be useful, for example, to clone polynucleotides that code for proteins to be expressed, to make polynucleotides to use as probes for detecting the presence of the desired mRNA in samples, for polynucleotide sequencing, or for other purposes.

Appropriate primers and probes for identifying ferulic acid esterase-specific genes, as well as xylanase sequences, from fungi and plant tissues are generated from comparisons of the sequences provided herein. For a general overview of PCR see PCR PROTOCOLS: A GUIDE TO METHODS AND APPLICATIONS, (Innis, M, Gelfand, D., Sninsky, J. and White, T., eds.), Academic Press, San Diego (1990). Reaction components are typically: 10 mM Tris-HCl, pH 8.3, 50 mM potassium chloride, 1.5 mM magnesium chloride, 0.001% gelatin, 200 µM dATP, 200 µM dCTP, 200 µM dGTP, 200 µM dTTP, 0.4 µM primers, and 100 units per mL Taq polymerase. Program: 96°C for 3 min., 30 cycles of 96°C for 45 sec., 50°C for 60 sec., 72°C for 60 sec, followed by 72°C for 5 min.

Polynucleotides may also be synthesized by well-known techniques as described in the technical literature. See, e.g., Carruthers, *et al.*, *Cold Spring Harbor Symp. Quant. Biol.* **47**:411-418 (1982), and Adams, *et al.*, *J. Am. Chem.*

Soc. **105**:661 (1983). Double stranded DNA fragments may then be obtained either by synthesizing the complementary strand and annealing the strands together under appropriate conditions, or by adding the complementary strand using DNA polymerase with an appropriate primer sequence.

Suitable sources for the ferulic acid esterase used in this invention include but are not limited to, *Neurospora crassa, Aspergillus spp.* and specifically, *Aspergillus niger.* The xylanase used in this invention can be derived from any suitable source including, but not limited to, *Trichoderma reesei* and *Aspergillus spp.*

B. Preparation of Recombinant Vectors

To use isolated sequences in the above techniques, recombinant DNA vectors suitable for transformation of plant cells are prepared. Techniques for transforming a wide variety of plant species are well known and described in the technical and scientific literature. See, for example, Weising, *et al., Ann. Rev. Genet.* **22**:421-477 (1988). A DNA sequence coding for the desired polypeptide, for example a cDNA sequence encoding the full length FAE1 protein, will preferably be combined with transcriptional and translational initiation and targeting regulatory sequences which will direct the transcription of the sequence from the gene in the intended tissues of the transformed plant under the desired conditions.

Promoters can be identified by analyzing the 5' sequences of a desired gene. Sequences characteristic of promoter sequences can be used to identify the promoter. Sequences controlling eukaryotic gene expression have been extensively studied. For instance, promoter sequence elements include the TATA box consensus sequence (TATAAT), which is usually 20 to 30 base pairs upstream of the transcription start site. In most instances the TATA box is required for accurate transcription initiation. In plants, further upstream from the TATA box, at positions -80 to -100, there is typically a promoter element with a series of adenines surrounding the trinucleotide G (or T) N G. Messing, *et al.,* in GENETIC ENGINEERING IN PLANTS, pp. 221-227 (Kosage, Meredith and Hollaender, eds. (1983)).

A number of methods are known to those of skill in the art for identifying and characterizing promoter regions in plant genomic DNA (*see, e.g.,* Jordano, *et al., Plant Cell* **1**:855-866 (1989); Bustos, *et al., Plant Cell* **1**:839-854 (1989); Green, *et al., EMBO J.* **7**:4035-4044 (1988); Meier, *et al., Plant Cell* **3**:309-316 (1991); and Zhang, *et al., Plant Physiology* **110**:1069-1079 (1996)).

In construction of recombinant expression cassettes of the invention, a plant promoter fragment may be employed which will direct expression of the gene in all tissues of a regenerated plant. Such promoters are referred to herein as "constitutive" promoters and are active under most environmental conditions and states of development or cell differentiation. Examples of constitutive promoters include the cauliflower mosaic virus (CaMV) 35S transcription initiation region, the 1'- or 2'- promoter derived from T-DNA of *Agrobacterium tumafaciens*, the actin and ubiquitin promoters and other transcription initiation regions from various plant genes known to those of skill. A particularly preferred constitutive promoter is the rice actin promoter (see, McElroy, *Plant Cell*, **2**:163 (1990)).

Alternatively, the plant promoter may direct expression of the polynucleotide of the invention in a specific tissue (tissue-specific promoters) or may be otherwise under more precise environmental control (inducible promoters). Examples of tissue-specific promoters under developmental control include promoters that initiate transcription only in certain tissues, such as leaves, roots or seeds.

In one aspect of the instant invention, expression of FAE occurs after the the plant has been cut, removed from the ground or ingested. Thus an appropriate promoter would be a senescence promoter. For example, *BFN1* has recently been shown to be a nuclease expressed in senescing leaves, Perez-Amador, *et al., Plant Physiol.* **122**:169 (2000). Similarly, SAG12, a cysteine protease is also found in senescing leaves (Noh & Amasino, *Plant Mol. Biol.* **41**:181 (1999). In a preferred embodiment, the promoter from the *gem* gene of *Festuca pratensis* is used to direct expression of FAE in senescing leaves.

In another aspect, the FAE would be expressed upon ingestion by a foraging animal. Exemplary promoters for this aspect would include Soybean Gmhsp 17.5 promoter and the leucine aminopeptidase (LAP) promoter. The

GMhsp promoter is from a heat shock protein gene and initiates expression if the temperature of the environment is increased. In the laboratory, an increase of 15°C for 2 hours is the preferred heat shock. However, in non-laboratory conditions suitable increases in temperature will occur in silos and in the rumen of animals that have ingested the plants of this invention. The LAP promoter initiates the expression of the FAE gene upon wounding of the plant. Such wounding would occur after cutting the plant or after mastication by a foraging animal. Tissue specific promoters that could be used in this invention include promoters of genes that are differentially expressed in the leaves of grasses. An example of a leaf specific promoter is the *rbcS* promoter of tomato (*Proc. Nat'l Acad. Sci. USA* **84**:7104 (1987)). This promoter normally regulates a gene determined to be important in photosynthesis.

For proper polypeptide expression, a polyadenylation region at the 3'-end of the coding region should be included. The polyadenylation region can be derived from the natural fungal gene, from a variety of other fungal or plant genes, or from T-DNA. These sequences are well known and readily available to those of skill in the art.

In addition to a promoter and poly A+ sequences, the preferred expression vectors of this invention also will contain signal sequences. These are polynucleotides found at the 5' and/or 3' ends of the coding region and serve to target expression of the gene to specific cellular organelles. These signal sequences can be both upstream or downstream of the coding region. Some preferred examples of upstream signal sequences include the barley aleurain sequence (Rogers, *Proc. Nat'l Acad. Sci. USA* **82**:6512 (1985) which targets vacuoles and the Aspergillus apoplast signal. This signal sequence targets expression to the apoplast.

In addition to targeting expression to specific organelles, it may be desireable to retain the expressed FAE in the Golgi or endoplasmic reticulum. The well known ER retention signal, KDEL, can be added to the 3' end of the coding polynucleotide.

The vector comprising the expression cassettes (*e.g.*, promoters and/or coding regions) of the invention will typically comprise a marker gene which

confers a selectable phenotype on plant cells. For example, the marker may encode biocide resistance, particularly antibiotic resistance, such as resistance to hygromycin, kanamycin, G418, bleomycin, or herbicide resistance, such as resistance to chlorosluforon or Basta.

C. Production of Transgenic Plants

DNA constructs of the invention may be introduced into the genome of the desired plant host by a variety of conventional techniques. For example, the DNA construct can be introduced directly to plant tissue using ballistic methods, such as DNA particle bombardment or the constructs may be introduced directly into the genomic DNA of the plant cell using techniques such as electroporation and microinjection of plant cell protoplasts. Alternatively, the DNA constructs may be combined with suitable T-DNA flanking regions and introduced into a conventional *Agrobacterium tumefaciens* host vector. The virulence functions of the *Agrobacterium tumefaciens* host will direct the insertion of the construct and adjacent marker into the plant cell DNA when the cell is infected by the bacteria.

See Dalton et al. (Co-transformed, diploid Lolium perenne (Perennial Ryegrass), Lolium multiflorum (Italian Ryegrass) and Lolium temulentum (Darnel) plants produced by microprojectile bombardment. Plant Cell Reports (1999) 18(9), 721-726) for exemplary methods for culturing and transformation of grasses.

Microinjection techniques are known in the art and well described in the scientific and patent literature. The introduction of DNA constructs using polyethylene glycol precipitation is described in Paszkowski, *et al., Embo J.* **3**:2717-2722 (1984). Electroporation techniques are described in Fromm, *et al., Proc. Natl. Acad. Sci. USA* **82**:5824 (1985).

Agrobacterium tumefaciens-mediated transformation techniques, including disarming and use of binary vectors, are well described in the scientific literature. See, for example Horsch, *et al., Science* **233**:496-498 (1984), and Fraley, *et al., Proc. Nat'l. Acad. Sci. USA* **80**:4803 (1983). US Patent 5,591,616 discloses *Agrobacterium* mediated transformation techniques in monocotyledons.

Ballistic transformation techniques are described in Klein, *et al., Nature*

327:70-73 (1987). In a preferred embodiment, a particle in-flow gun (PIG) is used to transform the plant cells of this invention.

Transformed plant cells which are derived by any of the above transformation techniques can be cultured to regenerate a whole plant that possesses the transformed genotype and thus the desired phenotype such as improved digestibility. Such regeneration techniques rely on manipulation of certain phytohormones in a tissue culture growth medium, typically relying on a biocide and/or herbicide marker which has been introduced together with the desired nucleotide sequences. Plant regeneration from cultured protoplasts is described in Evans, *et al.*, PROTOPLASTS ISOLATION AND CULTURE, HANDBOOK OF PLANT CELL CULTURE, pp. 124-176, Macmillian Publishing Company, New York, 1983; and Binding, *REGENERATION OF PLANTS, PLANT PROTOPLASTS*, pp. 21-73, CRC Press, Boca Raton, 1985. Regeneration can also be obtained from plant callus, explants, organs, or parts thereof. Such regeneration techniques are described generally in Klee, *et al., Ann. Rev. of Plant Phys.* **38**:467-486 (1987).

To determine the presence of or increase of FAE1 activity, an enzymatic assay can be used or an assay to measure increases and decreases in rates of fermentation. These assays are readily available in the literature and those of skill in the art can readily find them.

One of skill will recognize that other assays can be used to detect the presence or absence of FAE1. These assays include but are not limited to; immunoassays and electrophoretic detection assays (either with staining or western blotting).

The polynucleotides of the invention can be used to confer desired traits on essentially any plant. However, the main utility of this invention is in the improved digestibility of forage plants. Thus, it is envisioned the transgenic plants of this invention will include but not be limited to the following genera *Lolium, Festuca, Triticum, Avena,* and *Medicago*. The *FAE1* genes of the invention are particularly useful in the production of transgenic plants in the genus *Lolium.*

One of skill will recognize that after the expression cassette is stably incorporated in transgenic plants and confirmed to be operable, it can be

introduced into other plants by sexual crossing. Any of a number of standard breeding techniques can be used, depending upon the species to be crossed.

As mentioned above, the transgenic plants of this invention can be used as a foraging crop for animals, such as cattle, sheep, goats and horses. In addition, the methods of this invention can be used to transform any plant into which FAE expression is desired. For example, it is advantageous to break down cell walls during biomass conversion or during processing of plants for foodstuffs. This invention would help to achieve this goal more effectively and inexpensively.

The inventive methods herein may also be used to provide additional enzymes to enhance the availability of fermentable sugars in plants. Plant carbohydrates may be subject to further modification, either exogenously or endogenously, by the action of other enzymes. Such enzymes include, but are not limited to, endoglucanases, xylosidases and/or cellbiohydrolases. These enzymes may be provided either in an expression cassette provided for herein (i.e., endogenous) or applied to the plant cell walls (i.e., exogenous) to enhance the availability of mono- and/or di-saccharides.

Plants other than grasses may find a use in the present invention. For example, corn (or maize) is specifically contemplated to be useful. The grass Festuca is similar to maize in cell wall structure and therefore provides a good model of the ability to enhance fermentable carbohydrates in corn. Other useful plants contemplated for use in the present invention are Festuca, Lolium, Zea, Avena, Sorghum, Millet (tropical cereals), Miscanthus (a grass with potential for use as a biomass energy crop), Cenchrus, Dichanthium, Brachiaria and Paspalum (apomictic tropical range grasses) and Poa (Kentucky bluegrass).

Cell walls of forage grasses makes up 30-80% of forage dray matter representing a major source of energy for ruminants, but less than 50% of this fraction is digested by the animal. Conversion of low-value biomass to sugars and ethanol is also less than optimal due to the carbohydrate unavailability of the

feedstocks, including but not limited to bagasse, race straw, corn stover and corn fiber.

Ferulic and other hydroxycinnamic acids are ester linked to arabinosyl residues in arabinoxylans, and play a key role in crosslinking xylans to liginin, resulting in less degradable cell walls. Ferulic acid esterase (FAE) can release both monomeric and dimeric ferulic acid (FA) from arabinoxylans making the cell wall more susceptible to further enzymatic attack. Transgenic plants have been produced expressing an FAE gene following microprojectile bombardment of cell cultures. Measurements of the level of FAE activity from different vectors targeting FAE to the vacuole, ER and apoplast under constitutive or inducible (heat shock) promoters shows that at least for constitutive expression of vacuolar targeted FAE, the activity was highest in young leaves and increased along the leaf lamina. We also show that FAE expression results in release of monomeric and dimeric FA from cell walls on cell death and this was enhanced several fold by the addition of xylanase. An effect of FAE expression on the monomeric and dimeric cell wall ester linked ferulate content in comparison to control (non-transformed) plants is seen. Generally, the lower the levels of monomers and, in particular, dimers of hydroxycinnamic acids in leaves, the higher the digestibility and/or availability of complex carbohydrates for conversion.

Senescence is the terminal phase in leaf development and occurs without grouth or morphogenesis Therefore the metabolism/physiology of this stage of the leaf's lifespan can be targeted directly for alteration with minimal detrimental impact on early development. Senescence follows leaf maturity and is associated with the expression of specific genes. These genes and their controlling elements can be exploited to manipulate development, adaptation, productivity and quality traits in crop plants. There seems to be good conservation of senescence physiology across the range of higher plant species and thus these promoters are useful in the present invention.

The following preparations and examples are given to enable those skilled in the art to more clearly understand and practice the present invention. They should not be considered as limiting the scope and/or spirit of the invention, but

merely as being illustrative and representative thereof.

In the experimental disclosure which follows, the following abbreviations apply: eq (equivalents); M (Molar); μM (micromolar); N (Normal); mol (moles); mmol (millimoles); μmol (micromoles); nmol (nanomoles); g (grams); mg (milligrams); kg (kilograms); μg (micrograms); L (liters); ml (milliliters); μl (microliters); cm (centimeters); mm (millimeters); μm (micrometers); nm (nanometers); ° C. (degrees Centigrade); h (hours); min (minutes); sec (seconds); msec (milliseconds); Ci (Curies) mCi (milliCuries); μCi (microCuries); TLC (thin layer achromatography); Et (ethyl), Me (methyl).

Example 1

Preparation of Enzyme Encoding DNA Sequences

A genomic clone for FAE1 (see Figures 1-3) was used as the starting point for the preparation of an intronless FAE1 encoding DNA sequence. The sequence for the genomic clone is given in Figures 2 and 3. Separate fragments for both FAE exons were recovered by PCR from a 5.5kb EcoRI fragment of the genomic clone in pLITMUS28, and 'cDNA' created by overlapping PCR. See Figure 4.

Two 5' primers were used. FAE-S5 which amplifies the entire reading frame (including the Aspergillus *s*ignal), and FAE-N5 which amplifies only the mature protein (i.e. has *n*o signal). A number of codons are optimised (underlined in primer sequences below). The overlap product may be derived from either FAE-I5 (wild type) or FAE-I3 (conserved Ser changed to Ala) primers, allowing production of enzymatically inactive protein to check toxicity. As shown in Figure 5, overlapping of PCR products made with FAE-I5 and FAE-I3 creates two possible uninterrupted reading frames. If the complement to FAE-I5 serves as the template when recombined then the encoded protein retains the serine moiety and the esterase is functional (highlighted serine is at active site). If the FAE-I3 primer serves as the template the serine is replaced with an alanine and the esterase is inactivated (highlighted alanine in bottom amino acid sequence given in Figure 5).

Where possible, codon usage has been optimised in constructed reading frames (codon choice based on published barley preferences).

FAE-I5 (SEQ ID NO: ___)

GGCGCCGAGGGAGTGGCCGGTCACGGTCAGCGCGTAGTCC 40-mer

FAE-I3 (SEQ ID NO: ___)

CCGGCCACGCCCTCGGCGCCTCCCTGGCGGCACTC 35-mer

FAE-N5 (SEQ ID NO: ___)

CTAAAGCTTACCATGGCGGCCGCCTCCACGCAGGGCATCTCCGA 44-mer

FAE-S5 (SEQ ID NO: ___)

CTAAAGCTTAACATGAAGCAGTTCTCCGCCAA 32-mer

FAE-3 (SEQ ID NO: ___)

TCTAAGCTTGCGGCCGCGACCGGCCAGGTGCATGCGCCGCTCGTCATCCC 50-MER

Example 2

Preparation of Vectors

Vectors had the general structure shown in Figure 6.

A. Plant transformation vector series

Initial expression vectors were based on **pCOR105** [rice actin promoter - McElroy et al. MGG 231:150-160 (1991)] (Figure 7). pCOR105 Not and SstII sites were first destroyed [cut with NotI and SstI, followed by heat inactivation and T4 DNA polymerase treatment in the presence of dNTPs] using standard methods as described in Maniatis et al. or following the manufacturer's instructions for enzymes to simplify subsequent Not cassette manipulation and allow use of unique Sst site (see below).

The *nos* terminator from **pMA406** (Ainley & Key (1990) PMB 14:949-60) was amplified by PCR using primers TER5 and TER3 to generate a fragment with the following sequence (SEQ ID NO:___):

(Pst1) (Not 1)

(AGACTGCAGACCATGGCGGCCGCGKAACCACTGAAGGATGAGCTGTAAAG
AAGCAGATCGTTCAAACATTTGGCAATAAAGTTTCTTAAGATTGAATCCTGTT
GCCGGTCTTGCGATGATTATCATATAATTTCTGTTGAATTACGTTAAGCATGT
AATAATTAACATGTAATGCATGACGTTATTTATGAGATGGGTTTTTATGATTA
GAGTCCCGCAATTATACATTTAATACGCGATAGAAAACAAAATATAGCGCGC
AAACTAGGATAAATTATCGCGCGCGGTGTCATCTATGTTACTAGATCGATA
AGCTT CTA GATCT (where **K**=G or T)

(HindIII) (XbaI)

A redundancy in the TER5 primer (GCG**K**AA) creates fragments having either a stop codon (**T**AA) or glutamate codon (**G**AA) in one reading frame. The glutamate codon is in frame with a downstream KDEL motif.

The fragment and modified pCOR105 vector were cut with PstI and XbaI, according to manufacturers instructions, relevant fragments gel-purified, ligated with T4 DNA ligase and transformed into *E. coli*. Resulting clones were then sequenced to establish which TER5 alternatives were present.

Initial FAE expression vectors were then constructed from these vectors by inserting FAE-S5/FAE-3 PCR products (T4 DNA polymerase 'polished' in the presence of dNTPs, purified and digested with NotI, cloned into EcoRV and NotI digested vector) or FAE-N5/FAE-3 PCR products (purified and NotI digested, cloned into NotI digested and calf intestinal alkaline phosphatase treated vector).

The initial pCOR105-*nos* terminator clones were also modified by the addition of ALE-5/ALE-3 PCR products (encoding wild-type and modified barley aleurain signal peptides, see below for details). The products were 'polished' with T4 DNA polymerase in the presence of dNTPs, purified and cut with NotI, then cloned into EcoRV and NotI digested vectors. Addition of the ALE sequences creates a series of vectors which can express a reading frame inserted at the NotI or NcoI sites as a fusion to the barley aleurain signal, with or without vacuolar targetting motif, and with or without an ER retention motif. HindIII sites flanking the translation initiation codon and transcriptional terminator allow easy

movement of transcription units between expression vectors providing different promoter sequences. (See Figure 8 depicting the generic ALE-TER vector.)

Vector sequences were confirmed by sequencing. Two artifacts were found. Firstly, the redundant codon in TER5 was found to be AAA in one clone, which was subsequently used as the source of all KDEL fusions (ie peptide sequence is KPLKDEL, rather than EPLKDEL as designed). See Figure 9. Secondly, an additional base is found at the site of the redundant codon in one clone, creating a frameshifted terminal peptide (ETTEG, Figure 10) which was used as a control in some constructs.

Exploitation of the modular arrangement of signal peptides in the above vector series allowed various combinations of FAE and targeting motifs to be created using standard molecular biology procedures (i.e., restriction digest, purification of relevant fragments and ligation as appropriate). For example, the NotI fragment containing the FAE reading frame was inserted into the NotI site of the frameshifted clone described above to create vector **pTP3.1**. The native Aspergillus COOH-terminus was inserted into a FAE-S5/FAE-3 clone as a SphI (T4 DNA polymerase polished) – NcoI fragment from the FAE genomic clone (replacing the NotI (T4 DNA polymerase polished) – NcoI fragment), creating vector **pTP4a2**, which then encodes the entire, unmodified, Aspergillus FAE. Replacement of the SalI/XbaI fragment of pTP3.1 with that of pTP4a2 then created **pTP11.1**, which encodes FAE with a native Aspergillus COOH-terminus but a barley aleurain N-terminal signal.

Briefly, other vectors made in this series were; **pTP8.5**, the FAE NotI fragment inserted into the NotI site of an ALE-frameshifted COOH-terminus construct, aleurain N-terminus; **pTP5.1**, replacement of the native Aspergillus COOH terminus with a KDEL peptide (NotI/XbaI fragment exchange), Aspergillus N-terminal signal retained; **pTU4.4**, BamHI fragment of pTP11.1 replaces BamHI fragment of pTP5.1, creates FAE reading frame fused to heterologous N- and C-termini (aleurain signal and KDEL).

Vectors in which the aleurain vacuolar targeting motif NPIR was replaced by NPGR (found to be inactive in some plant assays) were created by replacing an EcoRV/NotI fragment with ALE PCR product which had been cut with AccI

(T4 DNA polymerase polished) and NotI (vectors **pTT5.5** and **pTT5.14**, Aspergillus COOH-terminus). The BamHI fragment of pTT5.5 was used to replace that of pTP5.1 to produce **pTU5**, creating an FAE reading frame fused to heterologous N- and C-termini (NPGR modification of aleurain signal and KDEL). The aleurain signal was also modified by PCR mutagenesis to remove the vacuolar targeting NPIR motif in its entirety (directed by primer ALECUT, which contains a NotI site to allow exchange of BglII/NotI fragments). NPIR deletion was created in this way in pTP11.1 (creating **pUA4.4**), and in pTP5.1 by exchange of BamHI fragments with pUA4.4 (creating **pUG4**).

Finally, PCR mutagenesis, using overlap of fragments generated by primers GLY3 and GLYB, was also used to alter a potential glycosylation site (asparagine codon changed to aspartate, as carried out for example in Chen, H.M., C. Ford & P. J. Reilly (1994) Biochem J 301 275-281 Substitution of asparagine residues in Aspergillus awamori glucoamylase by site-directed mutagenesis to eliminate N-glycosylation and inactivation by deamidation; see sequence data for exact change, vector **pTP10.1**).

PCR primers
TER-5 (SEQ ID NO:___)
AGACTGCAGACCATGGCGGCCGCG**KAA**CCACTGAAGGATGAGCTGTAAAG AAGCAGATCGTTCAAACATTTG 72-MER (The KDEL stop codon is underlined.)
TER-NOT (SEQ ID NO:___)
AAGACTGCAGACCATGGCGG 20-MER
TER-3 (SEQ ID NO:___)
AGATCTAGAAGCTTATCGATCTAGTAACATAGATGACACC
ALECUT (SEQ ID NO:___)
CTAGGCGGCCGCGCGGGAGGAGGCGACGGCGAC
GLYB (SEQ ID NO:___)
GAGGGTGTATTCGGTATCGAGTTGCAGGTTCGTATC
GLY3 (SEQ ID NO:___)
CTCGATACCCATTACACCCTCACGCCTTTCGA

B. Construction of different promoter vectors

Various promoters were used to optimize expression and to establish constitutive, heat-shock inducibility and senescence enhancement.

i. Rice actin promoter and 1st intron

Initial vectors (Figures 11 and 12) were constructed from pCOR105 which was subsequently found to contain a 5bp deletion relative to the published sequence which destroys the AccI site (GTAGGTAGAC, deleted bases underlined) and may affect splicing at the adjacent 3' site. The original rice actin sequence in this region (GTAGGTAG) was therefore restored using oligonucleotide **NCO-**ACT (CTCACCATGGTAAGCTTCTACC TACAAAAAAGCTCCGCA) by replacing the BglII/HindIII fragment with a PCR product, to produce vector **pPQ10.1**.

A rice repetitive element is present in the upstream region of the actin promoter used in **pCOR105**; as this may have unpredictable effects on vector expression it was removed from **pPQ10.1** by deletion of the KpnI/EcoRI fragment (end-filled with T4 polymerase and ligated following digest, restoring EcoRI but not KpnI), to produce vector **pGT6.** The HindIII fragment containing the FAE reading frame and nos terminator of **pTP3.1** (see Example 2A) was then inserted into pGT6 to produce construct **pJO6.3**.

ii. Soybean heat-shock promoter

A soybean heat shock promoter from a 23kD HSP was obtained from pMA406 (Ainley & Key (1990) PMB 14:949-60). This promoter when fused to β-glucuronidase (Jefferson et al 1987 EMBO J 6:3901-3907) had previously been shown to be inducible by a 10°C heat-shock and show stable expression for 24-48 hours (data not shown). β-glucuronidase fusions are a sensitive and versatile fusion marker in higher plants. The construction of the co-integration HS vectors is given below.

iii. Senescence enhanced expression (See1) promoter from Lolium multiflorum

The promoter and signal sequence (including NPIR motif) of the LSee1 gene was amplified from *Lolium multiflorum* cv Tribune with oligonucleotides SEE-NCO and SEE-VAC, and cloned as an Asp718/NotI replacement of the promoter region of vector **pTP11.1**. Following sequencing to screen for PCR artifacts, one of three identical clones was chosen (**pUB8.11**).

The See1 promoter from maize has been cloned previously and has

EMBL accession number is AX050343. See WO0070061.

The Lolium version of See1 was also cloned previously (Qiang Li (2000) Studies on leaf senescence and its genetic manipulation in *Lolium mutiflorum* PhD Thesis University of Wales, Aberystwyth) and has been shown to be senescence inducible when used to drive both β-glucuronidase and the Agrobacterium ipt gene.

An apoplast-targeted derivative was constructed by amplifying the Potato Protease Inhibitor (PPI) motif with primers PPI-AP6 and SEE-ATG, and cloning the product as an NgoMIV/NotI fragment into pUB8.11 (NgoMIV partial digest), to produce vector **pJQ5.2.** This vector has both the senescence induced promoter and the apoplast target sequence with the gene to be expressed inserted downstream of the apoplast sequence.

PCR Primers

SEE-VAC (SEQ ID NO: ___)

AACCATGGCGGCCGCGCGCTCGGTGACGGGCCGGAT

SEE-NCO (SEQ ID NO: ___)

TTCGGTACCATGGCCAGGTATAATTATGG

SEE-ATG (SEQ ID NO: ___)

CTGCGCCGGCGAGATGGMCGTGCACAAGGAG

C. Construction of targeting sequences

In order to examine whether or not the localization of the enzyme would have an effect on the phenolic acid content of the cell wall various signal sequences were utilized. The targeting sequences were added either to the N-terminus or to the C-terminus of the gene of interest.

i. N-terminal signal sequences

Six N-terminal signal sequences were utilized:

(a) The native Aspergillus end of FAE, plus excretion signal [apoplast localisation]

This is from the original clone and has the peptide sequence:

MKQFSAKHVLAVVVTAGHALAASTQGI.

(b) The mature Aspergillus end, with no excretion signal [cytoplasmic localisation]

Peptide sequence is MAAASTQGI (underlined motif is common to all constructs). Truncation of the signal sequence in (a) above was carried out by PCR with mutagenic primer FAE-N5.

(c) The barley aleurain signal, including intact NPIR motif [vacuole localisation]

The barley aleurain vacuolar signal sequence (See Figure 13; Swissprot database accession number P05167) was derived entirely from overlapping primers (ALE-5, ALE-3, ALE-CUT ALE-CAP-5 and ALE CAP-3). Following primer annealing at 37°C and extension with T4 DNA polymerase in the presence of dNTPs according to manufacturers instructions, PCR with flanking primers ALE-5 and ALE-3 was carried out. The product was 'polished' with T4 DNA polymerase, purified, digested with NotI and cloned into EcoRV/NotI digested pCOR105-nos terminator vector (see above). ALE-3 contains redundancies so that clones encoding NPIR or NPGR motifs may be recovered. Two versions of the signal, with and without the vacuole targeting motif, were produced, to give putative vacuolar NPIR and apoplast (NPGR) signal sequences.

PCR Primers

ALE-5 (SEQ ID NO: ___)

GGAATTCGTAGACAAGCTTACMATGGCCCACGCCCGCGTCCT 41-MER

ALE-3 (SEQ ID NO: ___)

TATCCATGGCGGCCGCGCGGTCGGTGACGGGCCGGMYCGGGTTGGAGTC GGCGAA 55-MER

ALE-CUT (SEQ ID NO: ___)

CTAGGCGGCCGCGCGGGAGGAGGCGACGGCGAC 33-mer

ALECAP-5 (SEQ ID NO: ___)

GCGACGGCGACGGCGGCCGTGGCCAGCACGGCGAGCGCCAGGAGGAGG ACGCGG 54-MER

ALECAP-3 (SEQ ID NO: ___)

TCGCCGTCGCCTCCTCCTCCTCCTTCGCCGACT 33-MER

(d) The barley aleurain signal, mutated to a NPGR motif [cytoplasmic localisation]

(e) The rat sialyl transferase golgi targeting motif [golgi localisation]

A Golgi targeting vector, **pJQ3.2**, was made by inserting a reading frame encoding the relevant rat sialyl transferase (RST) motif (See Figure 14. RST motif shown to function in plants by Boevink P, Oparka K, Cruz SS, Martin B, Betteridge A, Hawes C, (1998) PLANT JOURNAL 15 441-447 Stacks on tracks: the plant Golgi apparatus traffics on an actin/ER network) into vector pPQ10.1, and replacing the EcoRI/NotI promoter/signal fragment of **pJO6.3** with the fragment from this vector. Briefly, the RST motif was constructed by annealing oligonucleotides RST-F1A, RST-F1B, RST-F2A and RST-F2B, and amplifying the product with RST-5AD and RST-3A. This product was cloned and sequenced. Clones were found to have a deletion which was corrected by PCR with RST-RPT, followed by overlap-PCR and cloning of products.

PCR primers

RST-5AD (SEQ ID NO: ___)

ACTAAGCTTAAGGAGATATAACAATGATCCACACCAACCTCAA

RST-F1A **(SEQ ID NO: ___)**

TTCCATGATCCACACCAACCTCAAAAAGAAGTTCTCCCTCTTCAT

RST-F1B **(SEQ ID NO: ___)**

AGAGTGATCACGGCGAAGAGGAGGAAGACGAGGATGAAGAGGGAGAACTTCT TTT

RST-F2A **(SEQ ID NO: ___)**

TATAGATCTGCGTGTGGAAGAAGGGCTCCGACTACGAGGCCCTCACCCTCCAA GCCAAGGA

RST-F2B **(SEQ ID NO: ___)**

CATTTGGAACTCCTTGGCTTGGAGGGTG

RST-3A **(SEQ ID NO: ___)**

AACCATGGCGGCCGCCATTTGGAACTCCTTGGCT

RST-RPT **(SEQ ID NO: ___)**

TATAGATCTGCGTGTGGAAGAAGGGCTCCGACTACGAGGCCCTCACCCTCC AAGCCAAGGA

(f) otif [cytoplasmic localisation]

(g) The potato protease inhibitor II (PPI) apoplast motif [apoplast localisation]

An apoplast targeting reading frame was designed to encode the relevant potato protease inhibitor II (PPI) motif (See Figure 15) and cloned into **pJO6.3**, to produce vector **pJQ4.9.** Briefly, the PPI motif was constructed by annealing oligonucleotides PPI-AP1, PPI-AP2, PPI-AP3, PPI-AP4, PPI-AP5 and PPI-AP6, and cloning this product as a HindIII/NotI fragment into vector pPQ10.1; the EcoRI/NotI promoter/signal fragment of pJO6.3 was then replaced with the equivalent fragment from the modified pPQ10.1 vector.

PCR primers

PPI-AP1 **(SEQ ID NO: ___)**

GGAATTCGTAGACAAGCTTACMATGGMCGTGCACAAGGAGGT

PPI-AP2 **(SEQ ID NO: ___)**

GATCAGGAGGTAGGCWACGAAGTTWACCTCCTTGTGC

PPI-AP3 **(SEQ ID NO: ___)**

CCTACCTCCTGATCGTSCTCGGCCTCCTCTTGCTCGT

PPI-AP4 **(SEQ ID NO: ___)**

CCTTGGCGTCCACGTGCTCCATGGCGGAWACGAGCAAGAGGAG

PPI-AP5 **(SEQ ID NO: ___)**

GTGGACGCCAAGGCCTGCACCCKCGAGTGCGGCAACCTC

PPI-AP6 **(SEQ ID NO: ___)**

GGAATTCGCGGCCGCCGGGCAGATGCCGAAGCCGAGGTTGCCGCACT

ii. C-terminal end signal sequences

Four C-terminal signal sequences were utilized:

(a) Native Aspergillus end, [CTW] (vacuole and apoplast vectors)

This was derived directly from the genomic clone (see Example 1) as a Nco1-Sph1 fragment (Sph end filled with T4 polymerase) which replaces the

Nco1-Not1 region of a standard actin -FAE vector (Not1 end filled with T4 DNA polymerase).

(b) Expression vector linker alone [CTW-PVAAA] (plant optimised C-terminus for vacuole, golgi and apoplast vectors)

CTW is the peptide sequence of the Aspergillus FAE COOH end and is here provided by oligo FAE3. In this primer the reading frame is extended to provide the additional amino acids PVAAA which are partially encoded by the Not1 site used for cloning downstream signals see c) and d) below. Some COOH amino acids /motifs may affect compartment targeting, the PVAAA sequences are expected to be neutral in this respect while the native Aspergillus end may not be.

(c) Linker plus KPLKDEL [first K is primer artifact, intended to be E] {ER retention vectors)

These sequences are provided by primer TER5 introduced during PCR to generate the nos terminator fragment, and identified by sequencing within a specific clone . KDEL targeting has been demonstrated in plants by Denecke et al. ((1992) EMBO J 11: 2345-2355 Plant and mammalian sorting signals for protein retention in the endoplasmic reticulum contain a conserved epitope).

(d) Linker plus ETTEG [frameshift of (c)] (loss of ER retention - vacuole vectors)

These sequences are provided by primer TER5 introduced during PCR to generate the nos terminator fragment, and identified by sequencing within a specific clone (see Example 2A).

The KDEL signal is for ER retention, while others provide controls. A frameshift in the TER5 region [additional A] was used in subsequent constructs to destroy the ER KDEL retention signal.

The linker used in the above C-terminal targeting sequences was PVAAA.

D. Co-integration and co-transformation vectors.

Co-transformation vectors

A Hygromycin resistance gene driven by a CaMV345S promoter **(pRob5)** (35S-HYG-CMV in pUC18 (modified HYG, derived from pGL2) Bilang et al (1991)

Gene 100:247-50) was used for co-transformation experiments with **pTT3** and **pTP3.1, pJQ4.9, pJQ3.2, pJO6.3, pJQ5.2, pUB8.1 1** vectors.

Co-integration vectors

1. **Actin promoter constructs - pTR2.22, pTR6.1 , pTR8.1, pTR9.4, pTR7.1, pTT5.5 and 5.1.**

The CAMV35S-hyg region from pAJEB64TCA [a plant expression vector constructed by Andy Bettany at IGER containing CaMV-HYG from pTRA151 (Zheng et al 1991 Plant Physiol 97:832-835) (CaMV35S-HYG-tml terminator as clonable cassette in pUC4) cloned into KpnI site of pCOR105] was added as a HindIII fragment at the KpnI site (T4 polymerase blunt) of pTP4a2, in divergent orientation to FAE to create **pTR2.22**. The FAE/Nos HindIII fragment of this vector was replaced as follows in co-expression vectors . From pTP5.1 for **pTR6.1 ,** from pTP10.1 to **pTR8.1**, from pTP11.1 to **pTR9.4**. Signal sequences of FAE in pTR2.22 were replaced as HindIII/BglII fragments in **pTR7.1** (fragment from pT09.1). PCR products (ALE5/ALE-G) was digested with Acc1 and T4 polymerase, polished, followed by Not1 digest and cloning into EcoRV/ Not1 digested pTR2.22 to give clones **pTT5.5 and 5.1.**

PCR primer
ALE-G
TATCCATGGCGGCCGCGCGGTCGGTGACGGGCCGGCCCGGGTTGGAGTC
GGCGAA

2. **Actin promoter constructs -pUF1, pUA1K3, pUH4, pUH5, pUH6, pUH7, pUH8, pUH9.**

The HygR gene from pAJEB64TCA, driven by the CaMV promoter, was first cloned as an end-filled HindIII fragment at the end-filled XbaI site of pTP3.1, to give **pHOX3**. For ease of cloning the downstream HindIII site was destroyed to create **pUA1K3** and replacement of the FAE/Nos terminator HindIII fragment in this vector was carried out as follows. From pTP5.1 for **pUF1**, from **pTP11.1** for pUH4, from pTP8.5 for **UH5,** from pTT5 for **pUH6**, from pUA4.4 for **pUH7**, from pTU5 for **pUH8** and from pUG4 for **pUH9**.

3. Heat-shock promoter constructs - pUH10, pUH12, pUC5.11.

A co-transformation vector in which FAE is expressed from the soybean heat shock promoter was made by first modifying pMA406 to remove the nos terminator (BglII linearised and gel purified, KpnI digested, T4 DNA polymerase polished in the presence of dNTPs and recircularised), and then inserting the FAE HindIII fragment from pTP11.1, creating pTT3.1, which encodes the full aleurain signal and the native Aspergillus COOH-terminus.

Following assays of various constructs, co-integration vectors were constructed with FAE and HygR genes arranged in tandem.

The HygR gene from pAJEB-64-TCA, driven by the CaMV promoter, was first cloned as an end-filled HindIII fragment at the end-filled XbaI site of pTP3.1, to give pHOX3 and subsequently excised as a HindIII/SacI fragment (partial SacI digest, relevant sites found in flanking pTP3.1 sequences) which was cloned into the HindIII/SacI sites of pMA406, in tandem orientation (vector **pUH1a20**). FAE sequences were then cloned into the HindIII site of **pUH1a20** downstream of the heat -shock promoter (HindIII fragment from pTU5 for **pUH10**, HindIII fragment from pTT5 for **pUH12**). A pTP3.1 derivative was made by cloning the CaMV/HygR HindIII cassette from pAJEB-64-TCA in tandem orientation downstream of the FAE gene in pTP3.1, inactivating the middle HindIII site by partial digestion and end-filling, and excising the combined FAE/HygR cassette as a single HindIII fragment, which was inserted at the HindIII site in pMA406 to produce **pUC5.11**.

Example 3

Transformation of Plant Cells

Eight to ten weeks old embryogenic *F. arundinacea* and *L. multiflorum* suspension cultures were bombarded either with a single co-integration plasmid DNA vector containing FAE and hyg resistance genes, or with a co-transformation vector containing FAE and with plasmid pROB5 conferring hygromycin resistance (CAMV35S-hpt- nos) using a Particle Inflow Gun (PIG) (Finer et al. (1992) Development of the particle inflow gun for DNA delivery to

plant cells Plant Cell Reports 11:323-328) and 1.5-3.0 μm gold particles as in Dalton *et al* (Dalton et al. (1999) Co-transformed diploid *Lolium perenne (Perennial ryegrass), Lolium multiflorum* (Italian ryegrass*) and Lolium temulentum* (Darnel) plants produced by microprojectile bombardment. Plant Cell Reports. 18: 721-726) and Kuai et al (Regeneration of fertile transgenic tall fescue *(Festuca arundinacea)* plants with a stable highly expressed foreign gene. Plant Cell Tissue and Organ Culture (1999) 58:149-154). Transformants were selected with hygromycin (25 to 50mg /l) over a 10-12 week selection period at 25°C under continuous white fluorescent light (60 μE m^2 s^{-1}) and plants regenerated via somatic embryogenesis as in Dalton *et al* 1999, *supra*. Regenerated plants were screened for FAE activity on transfer to soil and expressing plants grown to maturity in a containment growth room at 18°C under 16h fluorescent lights (350 μE m^2 s^{-1}). Mature plants (6-8 weeks old) were re-assayed for FAE activity and fresh tissue harvested for Southern, Northern and Western analysis, and for self digestion analysis. The remaining tissue was freeze dried and powdered for cell wall structure analysis, in vitro-dry matter digestibility (IVDMD) determinations and for in-vitro gas production determinations of rates of tissue digestion.

Example 4

Targeting of Expression Product

To verify that the targeting sequences are effective in delivering the gene the targeting sequences were operably linked to a green fluorescent protein GFP. The vector constructs are shown in Figure 16. Cells were transformed by particle bombardment as in Example 3. Localization of the GFP could be visualized under a microscope 1 day after bombardment (i.e., shooting). See Figure 16.

Example 5

FAE1 activity

Plants regenerated from transformed cells showed FAE activity in all plant tissues tested. Cells were transformed as above under the direction of the ER and APO targeting sequences. FAE activity in transformed *Festuca arundinacea*

leaves of different ages was elevated compared to control (untransformed) plants. See Figures 17 and 18.

Similar results were seen with Lolium mutiflorum leaves at different ages transformed as above under the direction of vacuolar, ER and APO targeting sequence. See Figures 19 and 20.

FAE expression under a heat shock promoter can also be induced. (Data not shown.)

Thus, we have demonstrated FAE expression in Festuca and Lolium leaves under constitutive and HS promoters with effective FAE targeting to the vac, ER and apo.

FAE assay

FAE activity was determined in soluble extracts of fresh (or frozen at -70oC) leaves or cell cultures (0.5g) with 0.1M NaAc, pH 5.0 extraction buffer. Extracts were incubated with 24mM EF (ethyl 4-hydroxy-3-methoxycinnamate) or 1% FAXX as substrate, at 28°C for 24hrs and FAE activity calculated as the amount of ferulic acid released. FAE activity was also determined by measuring the release of monomeric and dimeric ferulic acid from self-digested leaf or cell culture samples. Fresh, or frozen, leaves or cell cultures (0.5g) were ground in 0.1M NaAc, pH5.0 extraction buffer in the presence and absence of xylanase (1000U **GC140**/sample) without added substrate and incubated at 28°C for 72hrs. Following incubation, and centrifugation, soluble extracts were loaded onto an activated reverse phase C18 µNova sep-pak column (Waters), eluted with 100% MeOH and the MeOH sample analysed by HPLC.

Example 6

Chemical Analysis of Cell Wall Extracts

Ester bound compounds were extracted from freeze dried powdered leaves or cell cultures (50 -100mg) with NaOH (5ml of 1M) followed by incubation at 25°C for 23hrs under N2. After centrifugation and acidification of the soluble extract with concentrated HCl, the extracted phenolics were loaded onto an activated reverse phase C18 µNova sep-pak column (Waters) and eluted with 100% MeOH. and the MeOH sample analysed by HPLC.

HPLC was carried out with methanol: 5% acetic acid either with a 35-65% MeOH gradient in 15min (FAE assay) or with a 30-70% MeOH gradient in 25 min (monomer and dimer cell wall components) at 2ml/min on a µNova Pak C18 8x10 RCM (Waters). Extracts were detected and quantified with a diode array detector (240-400nm Waters 996PDA) monitored at 280nm for aldehydes and 340nm for hydroxycinnamic acids. .

Levels of esterified monomeric and dimeric hydroxycinnamic acids in Festuca arundinacea plants expressing FAE under VAC, and ER and APO targeting sequences are reduced compared to control (untransformed) plants. The results can be seen in Figure 21 and 22, respectively. Thus, we show where this does not result in reduced cell wall phenolics in growing plants with vac targeting but does result in lower phenolics with ER and apo targeting. In addition,

Levels of esterified monomeric and dimeric hydroxycinnamic acids in Festuca arundinacea plants expressing FAE are not significantly reduced when FAE is VAC, targeting (Fig 21) which is as predicted for correct vacuolar targeting, but are significantly reduced, as predicted, in some plants when FAE was ER and APO targeted, compared to control (untransformed) plants. The results can be seen in Figure 22.

Example 7

In vitro dry matter digestibility. (IVDMD)

The *in vitro* dry matter digestibility (IVDMD) was estimated on 1.0 g dry weight of leaf or cell culture tissue using the pepsin/cellulase method of Jones and Hayward (The effect of pepsin treatment of herbage on the prediction of dry matter digestibility from solubility in fungal cellulase solutions. Journal of the Science of Food and Agriculture (1975) 26:711-718).

We show that the presence of FAE in the plants results in higher digestibility of the leaves. This may be due to internal FAE activity acting on normal cell walls with vacuole located FAE and to both FAE activity and the lower cell wall crosslinking with ER and apo targeted FAE (as also found with cell cultures).

End point digestibility as determined by IVDMD were higher in leaf tissue of some transformed plants of Festuca expressing FAE, compared to control (untransformed) plants. Examples are shown where vacuolar, ER or apoplast targeted FAE under a constitutive actin promoter have been effective at increasing IVDMD. Similar results were obtained with in leaves of Lolium, but were less pronounced.

The results can be seen in Figures 23 and 24.

Example 8

In vitro gas production measurements

In each experiment, 1.0-g samples of freeze dried powdered leaf tissue or cell culture were fermented in three 165-ml capacity serum bottles according to the pressure transducer technique of Theodorou et al. (Theodorou et al. (1994) A new gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds. Animal Feed Science and Technology 48: 185-197). Grab samples of rumen-digesta were taken at 8.00 h before the morning feeding from fistulated wethers fed grass hay, and transported to the laboratory in a pre-warmed (39°C) vacuum flask. The microbial inoculum and culture media were prepared as described by Theodorou et al. (1994). Each serum bottle received 10 ml of microbial inoculum, 85 ml of buffer and 4 ml of reducing agent.

At the end of the incubation period, (144h) the contents of each serum bottle were filtered through pre-weighed sintered glass funnels and freeze dried to constant weight. Dry matter loss was calculated as the difference between the dry weight of the sample pre- and post-incubation. Additionally, the concentration of volatile fatty acids (VFA) in the liquid fraction of the culture media at the end of the 144-h incubation period was determined by gas chromatography. A Chrompack CP 9000 chromatograph fitted with an automatic sampler (Chrompack 911) and a flame-ionisation detector, linked to a Dell PC with A1-450 integration software, was used for VFA quantification.

Gas production data were fitted to the model of France et al. (France, J., Dhanoa, M.S., Theodorou, M.K, Lister, S.J., Davies. D.R. and Isac, D. 1993. A model to interpret gas accumulation profiles associated with *in vitro* degradation of ruminant feeds. *Journal of Theoretical Biology.* 163: 99-111.) using the MLP (Ross,

G.J.S. 1987. *MLP, Maximum Likelihood Program Version 3.08*. Oxford Numerical Algorithms Group) package. The equation is in the form, $Y = A\{1 - e^{[-b(t-T) - c(\sqrt{t}-\sqrt{T})]}\}$ where Y is the cumulative gas production (ml), A is the asymptote (i.e. gas pool), T is lag time, and b (h^{-1}) and c ($h^{-0.5}$) are decay rate constants. A combined fractional rate (h^{-1}) of gas production (μ) was calculated as, $\mu = b + c/2\sqrt{t}$, where t is the incubation time (h).

It can be seen for *Festuca arundiancea* (denoted as BN in Figure 25) that cell cultures have a higher rate of digestion and cumulative gas production in the presence of FAE and that the addition of an exogenous xylanase further enhance the availability of fermentable carbohydrates. Similar results are found in FAE expressing cultures without added FAE . Fermentation rates are further increased compared with controls by the addition of exogenous FAE or xylanaase as these cultures expressing FAE have a reduced cell wall phenolic composition to controls Figures 26-28.

Example 9
FAE & xylanase transformed plants

Addition of exogenous xylanase (GC140) greatly increased FAE mediated release of phenolics from *Festuca* and *Lolium* leaves expressing *A. niger* FAE. See Figures 29-31 which show that phenolic release from leaf cell walls is increased in all FAE expressing plants on cell death and this is stimulated by xylanase irrespective of the targeting. Therefore expression of a fungal xylanase in plant cells is tested.

The FAE expression cassette is modified to comprise a fungal xylanase gene (either *T. reesei* or *A. niger*) to yield a FAE-xylanase expression cassette. The FAE-xylanase expression cassette is used to transform plant cells in a manner similar to those described in Example 3. The transformed cells are allowed to grow and are selected on an appropriate medium. The enzymes so expressed increase the availability of fermentable carbohydrates to a greater extent than the FAE expression cassette.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity and understanding, it will

be obvious that certain changes and modifications may be practiced within the scope of the appended claims.